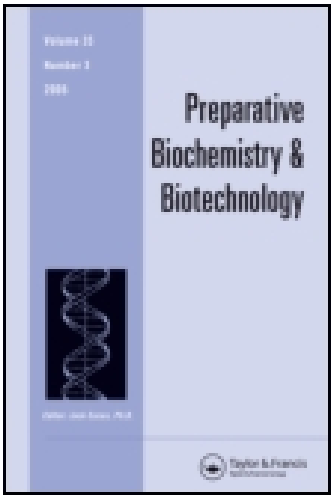

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H_2O_2 Determination by a Biosensor Based on Hemoglobin

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Abstract: The detection of hydrogen peroxide, H_2O_2, plays an important role in many fields including industry, environmental protection, and clinical control. Hydrogen peroxide can be toxic if ingested, inhaled, or by contact with the skin or eyes. Hemoglobin is a molecule with four electroactive iron hemes which can be used as an ideal model molecule for the study of electron transfer reactions of heme proteins and also for biosensing and electrocatalysis.

The present study describes the immobilization of hemoglobin on a Clark electrode surface to develop a novel electrochemical biosensor for the detection of hydrogen peroxide. The principle of the measurements was based on the electrocatalytic activity of the immobilized hemoglobin to the reduction of hydrogen peroxide. Hemoglobin was crosslinked with gelatine using glutaraldehyde and fixed on a pretreated teflon membrane. The optimum conditions for the biosensor were established. The most suitable hemoglobin and gelatin amounts and glutaraldehyde ratio were determined. Characterization studies of the biosensor, such as optimum pH and optimum temperature, were carried out. The repeatability experiments were done and the average value (x), standard deviation (S.D.), and variation coefficient (C.V.) were calculated. After the optimization and characterization studies the proposed biosensor was applied to determination of H_2O_2 in real samples.

Keywords: Biosensor, Clark electrode, Hemoglobin, Hydrogen peroxide

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INTRODUCTION

A hemoglobin molecule consists of four polypeptide chains: two alpha chains, each with 141 amino acids, and two beta chains, each with 146 amino acids.[1] A heme group is a flat ring molecule containing carbon, nitrogen, and hydrogen atoms, with a single Fe^{2+} ion at the center. Without the iron, the ring is called a porphyrin. In a heme molecule, the iron is held within the flat plane by four nitrogen ligands from the porphyrin ring.[2,3] The iron ion makes a fifth bond to a histidine side chain from one of the helices that form the heme pocket.[4]

Among various redox proteins, heme-proteins are mostly studied. It is well known that the catalytic activity of HRP is attributed to its iron heme group acting as the electroactive center. Since hemoglobin (Hb) contains four iron heme groups, it can be utilized as the HRP substitute in the detection of H_2O_2, by virtue of the low cost of Hb and the stable property of Hb in solution.[5] Electrochemical sensors for H_2O_2 based on the peroxidase-like activity of Hb incorporated in inorganic[6] or organic[7,8] films, porous silica networks,[9,10] and carbon nanotubes[11] have been fabricated. Moreover, the use of materials to incorporate heme proteins has also been reported that, including water insoluble surfactants[12,13] hydrogel polymers,[14–16] nanoparticles,[17] and lipids.[18]

The determination of hydrogen peroxide (H_2O_2) is of considerable importance in many different fields, such as in clinical, food, pharmaceutical, and environmental analyses. Consequently, the present study describes the immobilization of hemoglobin on a Clark electrode surface to develop a novel electrochemical biosensor for the detection of hydrogen peroxide. The principle of the measurements was based on the electrocatalytic activity of the immobilized hemoglobin to the reduction of hydrogen peroxide. Hemoglobin was crosslinked with gelatine using glutaraldehyde and fixed on a pretreated Teflon membrane.

First, the optimum conditions for the biosensor were established. In the optimization studies of the biosensor, the most suitable hemoglobin and gelatin amounts and glutaraldehyde ratio were determined. Characterization studies of the biosensor, such as optimum pH and optimum temperature, were carried out. The repeatability experiments were done and the average value (x), standard deviation (S.D.), and variation coefficient (C.V.) were calculated. After the optimization and characterization studies, the proposed biosensor was applied to determination of H_2O_2 in real samples.

EXPERIMENTAL

Apparatus

YSI 54 A, 57 A model oxygen meters, and YSI 5700 series dissolved oxygen (DO) probes (YSI Co Inc., Yellow Springs, Ohio, USA) were used. A water bath was used for preparation of bioactive material (Stuart Scientific Linear Shaker bath SBS 35, UK). All the measurements were carried out at a constant temperature using a thermostat (Haake JF, Germany). A magnetic stirrer (IKA-Combimag, RCO), and pH meter with an electrode (WTW pH 538, Germany) for preparing buffer solutions were used. The temperature was maintained constant in the reaction cell by circulating water at an appropriate temperature around the cell compartment during the experiment.

Procedure

Dissolved Oxygen Probe

In order to construct the biosensor, a dissolved oxygen probe was covered with high-sensitive Teflon membrane by using an *O*-ring.

Preparation of the Bioactive Layer Material

An appropriate amount of hemoglobin was weighed and dissolved with 200 μL working buffer (50 mM, pH 7, phosphate buffer). Then, an amount of gelatin was weighed and added to the hemoglobin solution. The mixture of hemoglobin and gelatin was incubated at 38 °C for 15 min to dissolve the gelatin.

Preparation of Biosensors

Hemoglobin-gelatin (200 μL) mixture for preparation of the biosensor was dispersed over the dissolved oxygen probe membrane surface and allowed to dry at 4 °C for 1 h. For crosslinking with glutaraldehyde, the probe carrying the bioactive layer was immersed in the glutaraldehyde solution and was allowed to wait for 5 min. Then, the biosensor was washed with distilled water to remove excess of the glutaraldehyde.

Measurement Procedure

The biosensor based on hemoglobin was put into the thermostatic reaction cell containing working buffer (pH 7, 50 mM sodium phosphate

buffer) and the magnetic stirrer was fixed at a constant speed. A few minutes later, dissolved oxygen concentration was equilibrated because of the diffusion of dissolved oxygen between the working buffer and the dissolved oxygen probe. At this time, hydrogen peroxide standard was injected into the thermostatic reaction cell. The dissolved oxygen concentration started to decrease and, a few minutes later, it reached the constant dissolved oxygen concentration. At this moment, dissolved oxygen concentration was recorded. Measurements were carried out by the change of dissolved oxygen concentration related to hydrogen peroxide added to the reaction cell.

RESULTS AND DISCUSSION

Optimization Studies

The following optimization and characterization studies were undertaken to achieve the best working conditions for hydrogen peroxide determination via the biosensor based on hemoglobin.

Effect of the Hemoglobin Amount

For the determination of the effect of hemoglobin amount on the biosensor response, the amount of hemoglobin on dissolved oxygen probe surface was changed as shown in Figure 1.

As is obvious from the figure, the optimum amount of the hemoglobin was 1.25 mg because of better linear range and higher response than the other ones. Besides, the biosensor which contained 5 mg of hemoglobin showed a slight decrease of signal. This probably was because of the diffusion barrier on the bioactive membrane constructed by a higher amount of hemoglobin.

Effect of the Gelatin Amount

In an investigation of the effect of differing gelatin amounts on the dissolved oxygen probe surface, experiments were carried out by keeping the amount of hemoglobin and percentage of glutaraldehyde constant and changing the amount of gelatin. Results obtained are given Figure 2.

As can be seen in Figure 2, the highest biosensor response was observed when 5 mg of gelatin was used. The biosensor signals were dramatically decreased when 2.5 mg of gelatin was used for the biosensor. This result could be related to the lower catalytic activity of a smaller amount of hemoglobin. Moreover, when the hemoglobin amount increased higher than 5 mg, the signal was again decreased. This result

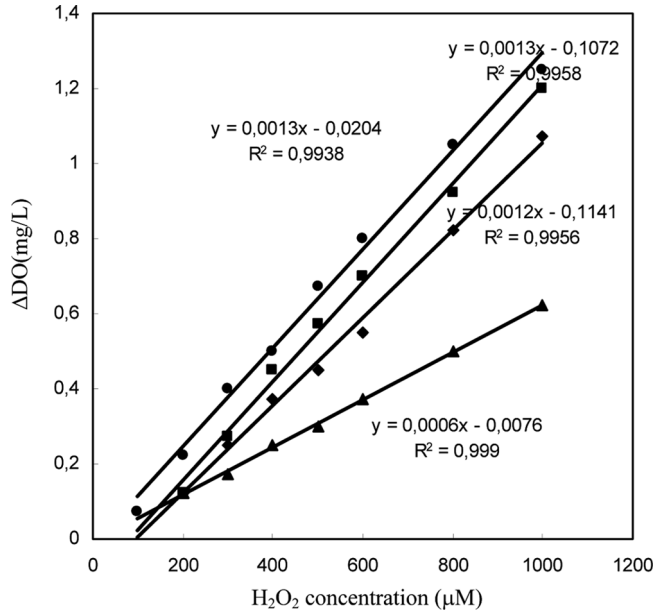

Figure 1. Optimization of hemoglobin amount [Hemoglobin amounts tested: -▲-▲-:0.5 mg, -●- ●-:1.25 mg, -■-■-:2.5 mg, and -◆- ◆-:5 mg. Gelatin amounts and glutaraldehyde percentages were kept constant as 5 mg and 2.5% respectively. Working conditions: pH 7, 0.05 M phosphate buffer, 35°C].

supported the assumption that the higher amount of the biosensor components affected the biosensor performance negatively because of the diffussion barrier.

Effect of the Glutaraldehyde Percentage

Effect of glutaraldehyde percentage on the biosensor was also investigated. The biosensors were prepared using varying concentrations of glutaraldehyde, as described in the procedure section. The results of the studies are given in Figure 3. The results showed that the best biosensor signals were obtained with the glutaraldehyde at 2.5%. Similarly, a diffusion barrier effect can be considered in the case of glutaraldehyde concentrations higher than 2.5%.

Optimum Temperature

The effect of temperature on the hemoglobin biosensor was investigated. Temperatures of 20, 25, 30, 35, 40, 45, and 50 °C were studied as working

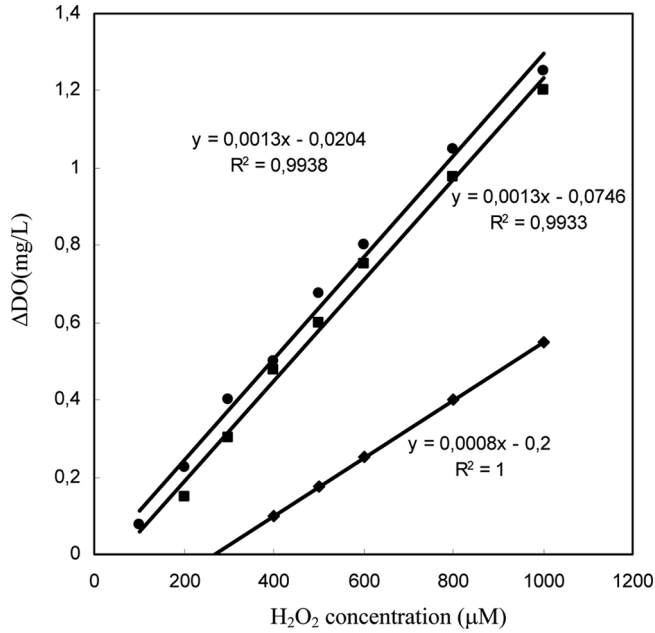

Figure 2. Optimization of gelatin amount [Gelatin amounts tested: -◆-◆-: 2.5 mg, -●-●-:5 mg, and -■-■-:7.5 mg. Hemoglobin amounts and glutaraldehyde percentages were kept constant as 1.25 mg and 2.5%, respectively. Working conditions: pH 7, 0.05 M phosphate buffer, 35°C].

temperatures. As can be clearly understood from the Figure 4, the temperature influenced the biosensor response, considerably.

The highest biosensor response was observed at temperatures of 35 and 40 °C. Above and below of these temperatures, there was a decrease in the biosensor response of about 15–20%. Although, at the temperature of 40 °C, a response of 100 % was obtained, in order to prevent possible denaturation of the bioactive membrane of the biosensor, the temperature of 35 °C was accepted as working temperature in all studies.

Optimum pH

As a rule, the maximum of the enzyme activity is evaluated as most appropriate for the substrate. Indeed, the pH dependence of the observed effect often corresponds to that of the response of a biosensor. This rule is valid in catalytic activity of hemoglobin because it contains a heme group. The results are shown in Figure 5.

Appropriate buffer systems (50 mM) were used for these studies. For pHs between 4 and 6 the citrate buffers were used. The other buffer

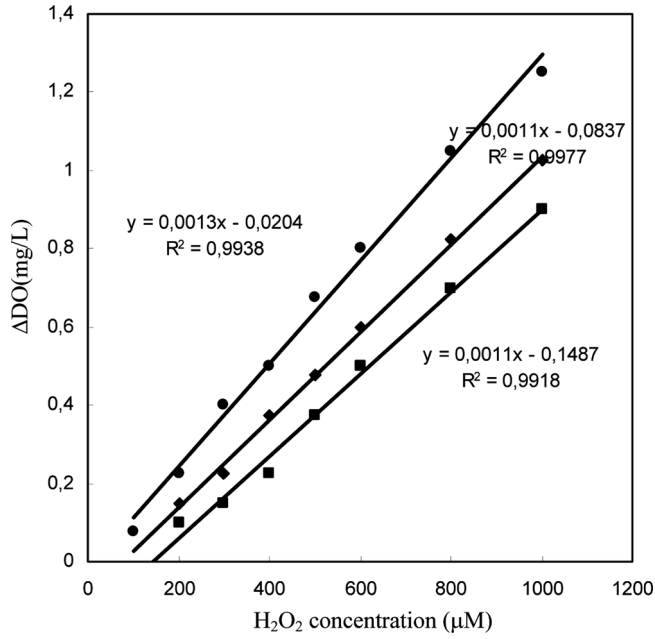

Figure 3. Optimization of glutaraldehyde percentage [Glutaraldehyde percentages tested: -◆-◆-:1.25%, -●-●-:2.5%, and -■-■-:5%. Hemoglobin and gelatin amounts were kept constant as 1.25 mg and 5 mg, respectively. Working conditions: pH 7, 0.05 M phosphate buffer, 35°C].

systems higher than pH 6 were phosphate buffers. As can be seen from the figure, the optimum pH of the biosensor was 7.

Characterization Studies

Calibration Graph for H_2O_2

After optimum conditions of the biosensor were investigated, a calibration graph of the hemoglobin biosensor for H_2O_2 was plotted. R^2 value was very good (0.9923). A linear range was observed between 100 and 1,000 μM H_2O_2. This plot is shown in Figure 6.

Repeatability of the Signals

The repeatability of the biosensor was also studied for 600 μM H_2O_2 standard concentration ($n = 10$). The average value (x), the standard deviation, and variation coefficient were calculated as 590 μM, ± 16.2 μM,

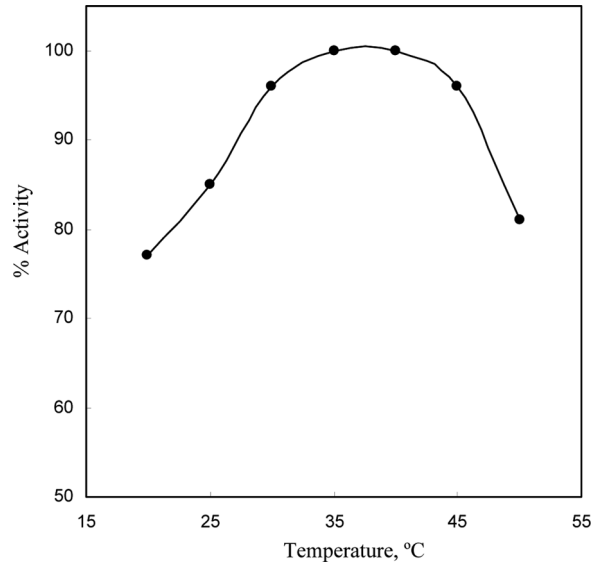

Figure 4. Optimum temperature [Biosensor components were kept constant as optimum. Working conditions: pH 7, 0.05 M phosphate buffer, hemoglobin standard used in the experiments was 600 μM].

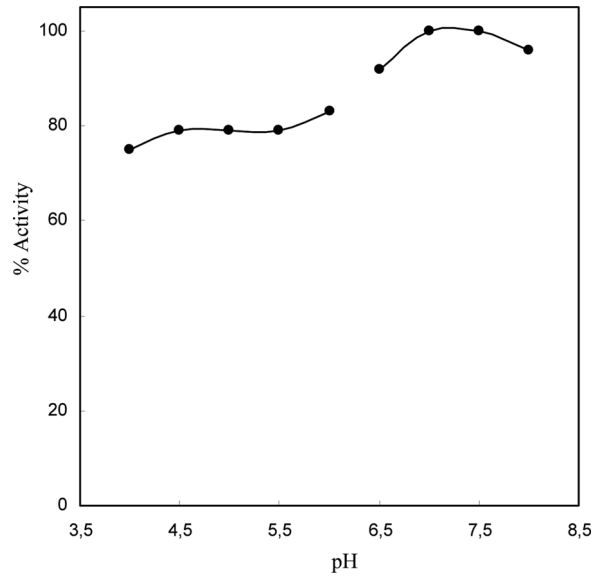

Figure 5. Optimum pH [Biosensor components were kept constant as optimum. Working conditions: 0.05 M citrate and phosphate buffers with different pHs, hemoglobin standard used in the experiments was 600 μM, 35°C].

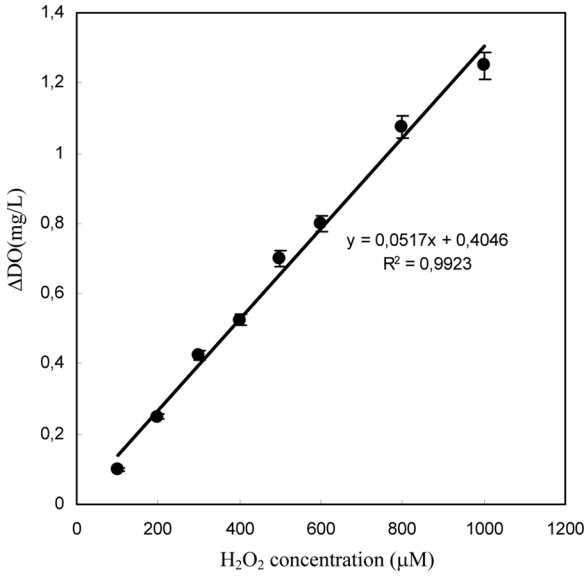

Figure 6. H_2O_2 calibration graph [Hemoglobin and gelatin amounts, and glutaraldehyde percentage were 1.25 mg, 5 mg and 2.5%, respectively. Working conditions: pH 7, 0.05 M phosphate buffer, 35°C].

and 2.7%, respectively. The biosensor based on hemoglobin presented a perfect performance for hydrogen peroxide.

Sample Analysis

The biosensor based on hemoglobin was examined by the determination of H_2O_2 in milk. The samples were not pretreated. A concentration of H_2O_2 spiked milk samples were determined with the use of the biosensor. The results are shown in Table 1.

Good agreement between the measurements was achieved. These results indicate that the proposed biosensor can be applied successfully for determination of H_2O_2.

Table 1. H_2O_2 determination in certain milk samples spiked with H_2O_2

Sample	Added[a] (μM)	Found[a] (μM)	Recovery (%)
1	600	611	+1.8
2	600	611	+1.8
3	600	592	−1.3

[a]Mean of three determinations.

CONCLUSION

The results presented in this work demonstrate that the hemoglobin biosensor is feasible for the detection of H_2O_2. The biosensor is readily prepared from inexpensive and commercially available materials through a general method. At the end of this work, the biosensor showed very good performance for detection of H_2O_2. A wide linear response range, between 100 and 1000 μM of H_2O_2, was verified. The simplicity in the bioactive material immobilization demonstrated a considerable economic advantage because of low cost. Finally, real sample analyses showed that the biosensor could be used for H_2O_2 determination in real samples.

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